

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Re:	Application No. 10/559,996	)	<i>Confirmation No. 4587</i>
Filed:	October 4, 2006	)	
Applicants:	Ralf KRAHMER et al.	)	This Declaration was electronically filed on September <u>  </u> 2008 using the USPTO's EFS-Web.
Title:	REAGENTS FOR MODIFYING BIOPHARMACEUTICALS, THE USE, AND PRODUCTION THEREOF	)	
Art Unit:	1654	)	
Examiner:	Ronald T. Niebauer	)	
Attorney Docket:	5942/87136	)	
Customer No.:	22242	)	

Commissioner for Patents  
P.O. Box 1450  
Alexandria, VA 22313-1450

DECLARATION OF RALF KRAHMER UNDER 37 C.F.R. SEC 132

Sir:

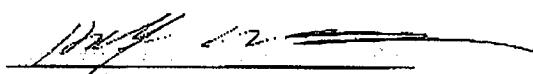
I, Ralf Krahmer, declare as follows.

1. I am one of the inventors of the above identified application.
2. I have conducted experiments to synthesize compounds which represent compounds being claimed. A description of those experiments and results are attached.
3. The experimental data is directed to further Examples 17-22, which show two groups of formula IIa and wherein n is 4.

Application No. 10/559,996  
DECLARATION OF RALF KRAHMER

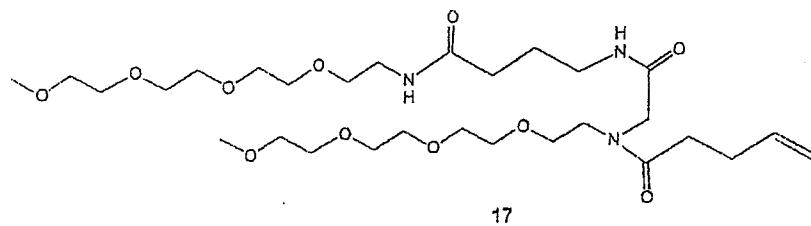
The undersigned, being warned that wilful false statements and the like are punishable by fine or imprisonment, or both (18 U.S.C. §1001) and may jeopardize the validity of the application or any patent issuing thereon, hereby declares that the above statements made of my own knowledge are true and that all statements made on information and belief are believed to be true.

Date: 17.09.2008

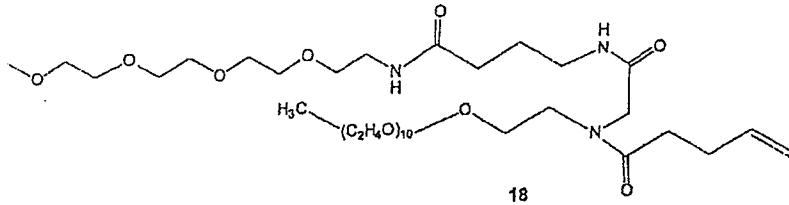
  
Ralf Krahmer

### Further experimental examples

According to the described possibility of using an acid component which has a protecting group character and is then split by forming a secondary amine, compounds **17** and **18** are shown.



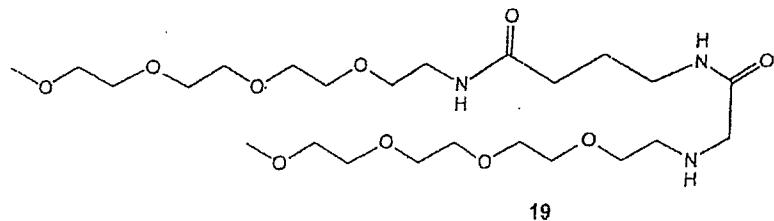
MS (ES+):m/z: 622.5 [M+H]<sup>+</sup>; 644.5 [M+Na]<sup>+</sup>; C<sub>29</sub>H<sub>55</sub>N<sub>3</sub>O<sub>11</sub>.



MS (ES+):m/z: 930.7 [M+H]<sup>+</sup>; 952.7 [M+Na]<sup>+</sup>; C<sub>43</sub>H<sub>83</sub>N<sub>3</sub>O<sub>18</sub>.

*iodine*

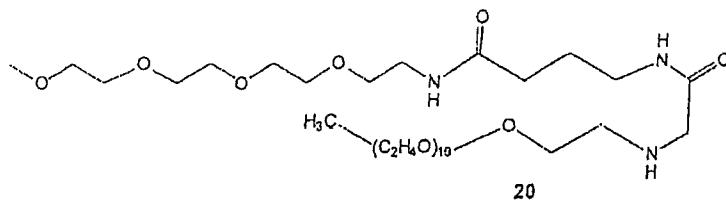
- By reacting with *iodine*, the pentenoyl protecting group is then removed by forming secondary amines **19** and **20**.



MS (ES+):m/z: 540.4 [M+H]<sup>+</sup>; C<sub>24</sub>H<sub>49</sub>N<sub>3</sub>O<sub>10</sub>.

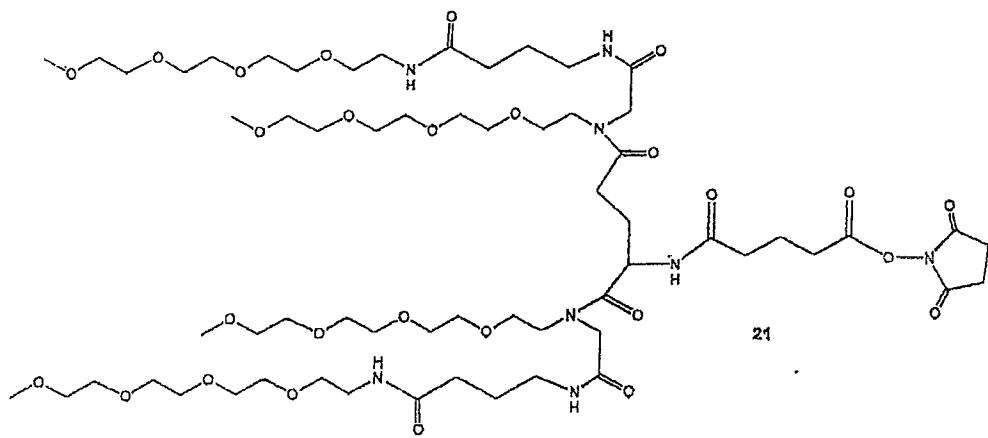
<sup>1</sup>H-NMR (300 MHz, CDCl<sub>3</sub>): δ = 1.79 (CH<sub>2</sub>, 2H); 2.17 (CH<sub>2</sub>, t, 2H); 2.52 (CH<sub>2</sub>, bs, 2H); 2.72 (CH<sub>2</sub>, t, 2H); 3.23 (2xCH<sub>3</sub>, s, 6H); 3.16-3.8 (m, 32 H), 6.77 (NH, bs, 1H); 7.52 (NH, bs, 1H); NH glycin exchanges, no signal.

<sup>13</sup>C-NMR (75 MHz, CDCl<sub>3</sub>): δ = 25.93; 33.68; 38.30; 39.23; 49.30; 52.16; 59.01; 59.04; 69.84; 70.15; 70.19; 70.29; several signals O-CH<sub>2</sub> at 70.44 to 70.70; 71.92; 172.04; 172.63.

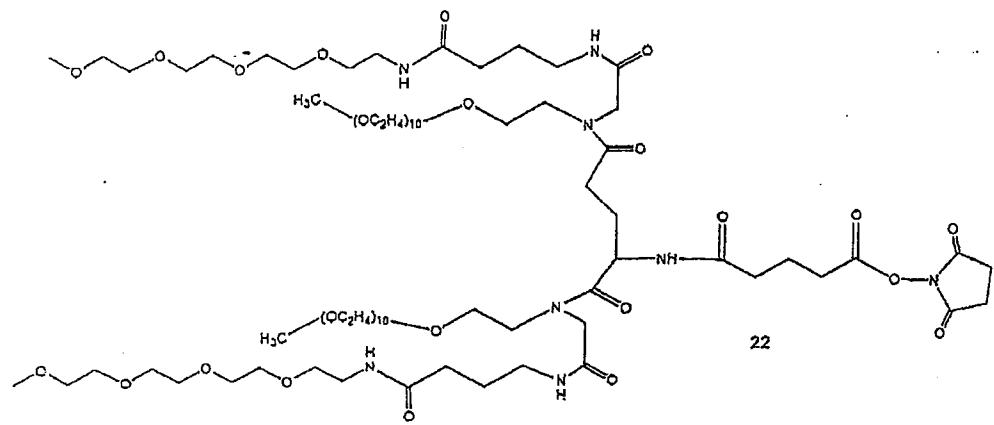


MS (ES+):m/z: 848.7 [M+H]<sup>+</sup>; 870.7 [M+Na]<sup>+</sup>; C<sub>38</sub>H<sub>77</sub>N<sub>3</sub>O<sub>17</sub>.

Compounds **19** and **20** are coupled to Boc-protected glutamic acid within the framework of a peptide coupling by using DCC. Acid-catalyzed cleavage of the Boc-group releases the α-amino function which is then modified by using glutaric acid anhydride. The active esters **21** and **22** are obtained according to the general description for **15**.



MALDI-MS:  $m/z$ : 1423.6  $[M+Na]^+$ ;  $C_{62}H_{112}N_8O_{27}$ .



MALDI-MS:  $m/z$ : 2024.3  $[M+Li]^+$ ;  $C_{90}H_{168}N_8O_{41}$ .

## Description of the experiments (Biochemistry)

### Description of the experiments regarding antigenicity

The antigen detection of L-asparaginase and modified L-asparaginase by specific anti-L-asparaginase antibodies was determined by means of competitive ELISA. Microtiter plates were coated with anti-L-asparaginase antibodies (US Bio, US). Afterwards, increasing concentrations from 0 to 35pmol/mL of modified and/or for the control of non-modified L-asparaginase were incubated together with an L-asparaginase marked with horseradish peroxidase of constant concentration. Excess antigen was washed from the microtiter plate. The quantification of the bonded competitor was effected by the proof of horseradish peroxidase with TMB as a substrate. The reaction was stopped with sulfuric acid after 10-15 min. The absorption was measured at 450 nm with 620 nm as reference filter and applied on a diagram dependent on the antigen concentration. The higher the antigen detection by ~~a~~ specific antibody, the lower the absorption values at 450 nm. ~~the~~

### Figures

#### Figure 6

Comparison of the influence of a modification of asparaginase with substance 21 and/or with linear PEG-SS-5000 (Sunbio Inc., Korea) on the stability of L-asparaginase over chymotrypsin. The stability of L-asparaginase is significantly increased <sup>compared to</sup> ~~over~~ <sup>substance</sup> chymotrypsin by modification with <sup>21</sup> and is comparable with the stability of L-asparaginase modified with PEG-SS-5000. Despite the lower molecular weight the branched reagent surprisingly effects a similar protection <sup>against</sup> ~~over~~ proteolysis as the linear PEG-SS-5000 which is 2.5 times the size.

**Figure 7**

Comparison of the antigen detection in L-asparaginase modified with a substance **22** and/or linear PEG-SBA-2000 (molecular weight of 2000 Da, respectively) by specific anti L-asparaginase antibodies by means of ELISA. The PEGylation degrees of the tested L-asparaginase preparations were equal. L-asparaginase modified with substance **22** was bonded considerably worse by the specific antibodies than L-asparaginase modified with linear PEG of the same molecular weight. The *in vitro* antigenicity of L-asparaginase is <sup>ied</sup> considerably reduced when modifying with a branched compound of the invention compared to the already known compound PEG-SBA-2000. *unexpectedly*

**Figure 8a)**

MALDI mass spectrometric analysis of the invention according to substance **22** (corresponds to the term CES0218). It is possible by means of the present invention to produce polymer-like, branched substances which are monodisperse and thus for the most part result in one single signal with  $m/z = 2024.3 [M+Li]^+$  from a mass spectrometric point of view. This is of great importance for the commercial application of reagents for the modification of therapeutic active substances.

**Figure 8b)**

MALDI mass spectrometric analysis of a commercially usual, polydisperse PEG-SBA-2000 PEGylated substance which is used for the modification of active substances. Corresponding to a Gauss-distribution, PEG-SBA-2000 consists of an undivisible mixture of PEG chains which always differ by 44 Da in mass corresponding to an ethylene glycol unit.

## Examples

### Example B4

1 Production of a conjugate from a compound of the invention according to substance 21 and L-asparaginase.

20 µL of substance 21 dissolved in dimethyl sulfoxide (25 mg/mL) was added to 180 µL of a solution of L-asparaginase (0.5 mg/mL) in sodium carbonate buffer (pH 8.5 to 9.5). The reaction mixture was incubated at 25°C and 300 rpm for 1 h on a thermomixer. The reaction was stopped by adding 4 µL of a 3 M ethanolamine solution and incubated at 25°C for another period of 10 min. Then, the excess substance 21 was removed by means of filtration in centrifuge filtration units (10 kDa cut-off) using water as rinsing liquid.

A PEGylation degree of 30% of this L-asparaginase preparation was determined.

Production of a conjugate from PEG-succinimidyl succinate 5000 Da (PEG-SS-5000) and L-asparaginase.

30 µL of PEG-SS-5000 dissolved in dimethyl sulfoxide (50 mg/mL) was added to 170 µL of a solution of L-asparaginase (0.5 mg/mL) in sodium carbonate buffer (pH 8.5 to 9.5). The reaction mixture was incubated at 25°C and 300 rpm for 1 h on a thermomixer. The reaction was stopped by adding 4 µL of a 3 M ethanolamine solution and incubated at 25°C for another period of 10 min. Then, the excess PEG-SS-5000 was removed by means of filtration in centrifuge filtration units (10 kDa cut-off) using water as rinsing liquid.

A PEGylation degree of 37% of this L-asparaginase preparation was determined.

The two preparations of modified L-Asparaginase were compared with each other with regard to their stability over chymotrypsin (Figure 6).

### Example B5

Production of a conjugate from a compound of the invention according to substance 22 and L-asparaginase.

5 µL or 10 µL of substance 22 dissolved in dimethyl sulfoxide (75 mg/mL) was added to 190 µL of a solution of L-asparaginase (0.5 mg/mL) in sodium carbonate buffer (pH 8.5 to 9.5). The reaction mixture was incubated at 25°C and 300 rpm for 1 h on a thermomixer. The reaction was stopped by adding 4 µL of a 3 M ethanolamine solution and incubated at 25°C for another period of 10 min. Then, the excess substance 22 was removed by means of filtration in centrifuge filtration units (10 kDa cut-off) using water as rinsing liquid.

A PEGylation degree of 17 or 22%, respectively, of this L-asparaginase preparation was determined.

| **¶** Production of a conjugate from a PEG-butanoic acid of 2000 Da (PEG-SBA-2000, Nektar Inc., USA) and L-asparaginase.

2.5 µL or 5 µL 30 µL of PEG-SBA-2000 dissolved in dimethyl sulfoxide (75 mg/mL) was added to 190 µL of a solution of L-asparaginase (0.5 mg/mL) in sodium carbonate buffer (pH 8.5 to 9.5). The reaction mixture was incubated at 25°C and 300 rpm for 1 h on a thermomixer. The reaction was stopped by adding 4 µL of a 3 M ethanolamine solution and allowed to stand at 25°C for another period of 10 min. Then, the excess PEG-SS-5000 was removed by means of filtration in centrifuge filtration units (10 kDa cut-off) using water as rinsing liquid.

| A PEGylation degree of 17% or 22%, respectively of this L-asparaginase preparation was determined.

The two preparations of modified L-asparaginase were compared with each other with regard to their detection by specific anti L-asparaginase antibodies by means of competitive ELISA (Figure 7).

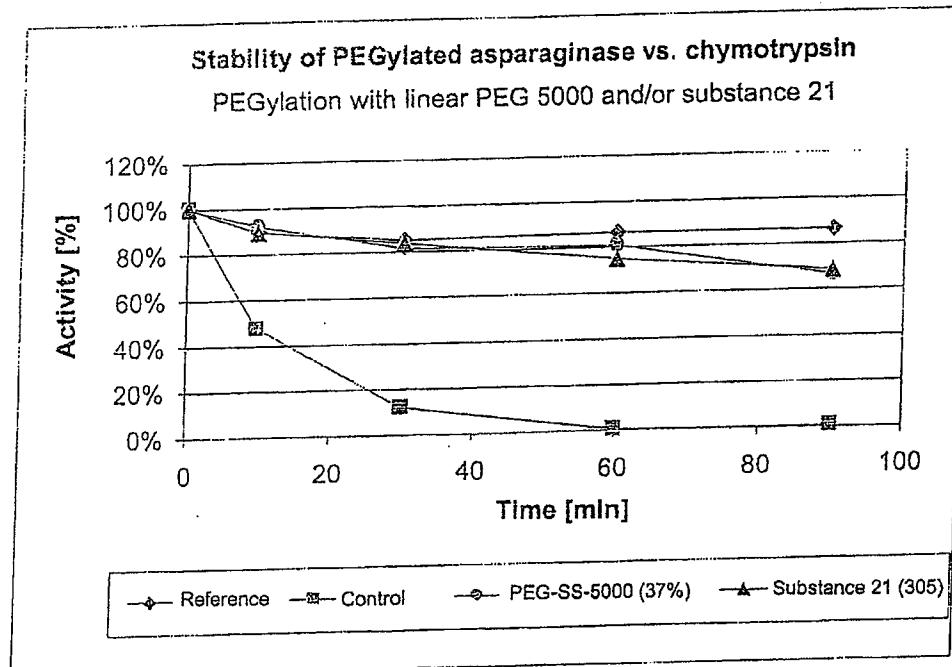


Figure 6

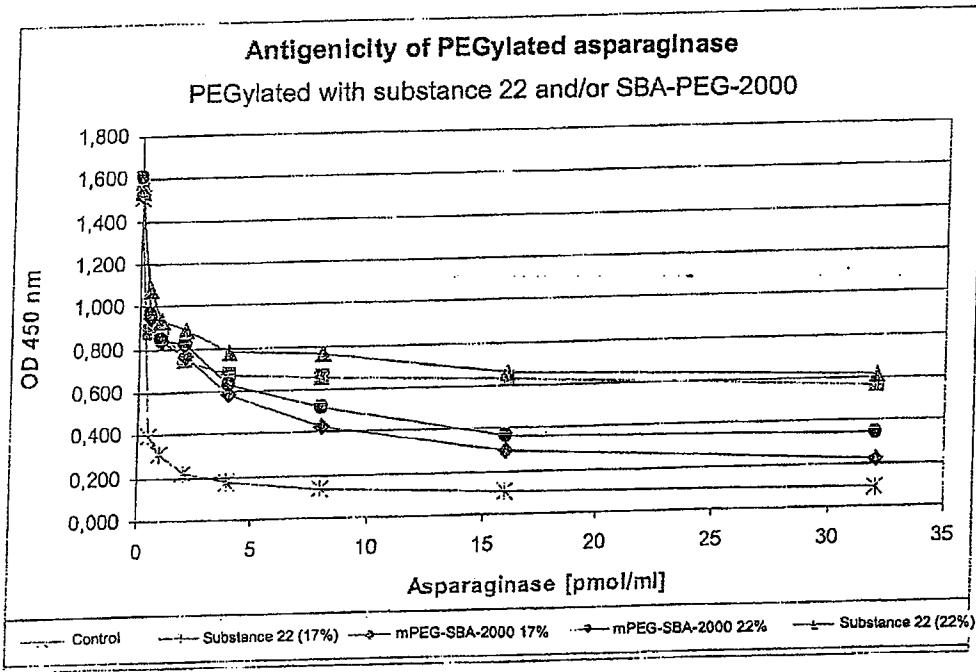


Figure 7

Comment 1 CES0218\_B2-4\_chrom1#5-21.Fa\_Celares  
Comment 2 MALDI-MS, Matrix: Dithranol + Li

